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<b>(54) Title:</b> LIGANDS AND BINDING PARTNER FOR TREATMENT AND DIAGNOSIS OF INFLAMMATORY CONDITIONS  <b>(57) Abstract</b> <p>Ligands bind a binding partner found on the surface of activated T cells and epithelial cells of inflamed gut, but not epithelial cells of normal gut. The binding partner is recognised by an antibody, denoted 4D5.4 and available from deposit ECACC 91111901, and also a naturally occurring ligand found in the body. Fetal gut displaying symptoms of an inflammatory disorder was treated with 4D5.4 and a reduction in the displayed enteropathy was achieved. The binding partner recognised by 4D5.4 antibody is involved in T cell activation by antigens and mitogens. The antibody, the binding partner recognised by it, other ligands which bind the binding partner recognised by it, and fragments and derivatives of all these are useful in the diagnosis and treatment of inflammatory conditions, mediated by T cells, for example disorders of the gut such as Crohn's disease.</p>		

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LIGANDS AND BINDING PARTNER FOR TREATMENT AND DIAGNOSIS  
OF INFLAMMATORY CONDITIONS

The present invention relates to ligands, eg  
5 antibodies, and a binding partner useful for treatment  
and diagnosis of inflammatory conditions. The present  
invention also relates to uses of the ligands and binding  
partner and methods of their making.

In particular, the present invention relates to a  
10 molecule on the surface of cells designated herein as the  
4D5.4 receptor and to the 4D5.4 hybridoma producing an  
antibody which reacts strongly with epithelial cells from  
inflamed tissue eg human guts, and also with lymphocytes  
from sites of inflammation, eg in the gut. This  
15 reactivity is quite specific, as the 4D5.4 receptor as  
detected by the antibody, is expressed only very weakly,  
if at all, on normal gut epithelial cells. In addition,  
it is expressed on a number of lymphoid cell lines, and  
on normal T lymphocytes activated in vitro with a  
20 polyclonal mitogen such as phytohaemagglutinin.

Inflammation is the body's response to tissue  
injury. The response may be acute, in most cases  
resulting in repair of the destruction and tissue  
regeneration, thus limiting the damage to the organism.  
25 Sometimes, however, the inflammatory response becomes  
chronic and this can result in an inflammatory condition  
or disease with tissue degeneration and significant  
morbidity. The damage induced by this latter form of  
inflammation is mediated by an influx of activated T  
30 lymphocytes and macrophages. These cells release a  
number of chemical mediators some of which are themselves  
toxic, others of which attract more cells into the site  
of inflammation.

Intestinal damage as a result of allergy and  
35 infection is a major cause of morbidity in man and  
animals (e.g. Ferguson, A 1976. Coeliac disease and  
gastrointestinal food allergy. In Immunological Aspects  
of the Liver and Gastrointestinal Tract. Anne Ferguson  
and R.N.M. McSween, editors. MTP Press Ltd, Lancaster:  
40 153-202). In intestinal allergy, the most commonly seen

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lesions are small intestinal villous atrophy and crypt cell hyperplasia resulting in malabsorption due to a decrease in the intestinal absorptive surface and decreased digestive enzyme levels in epithelial cells.

5 Crohn's disease is an example of an inflammatory disorder of the intestine. The disease is of unknown aetiology and may start in the teens and early twenties. The terminal ileum is most frequently affected, but any part of the gastrointestinal tract can be involved. In  
10 affected areas, the wall of the intestine is thickened and the lumen narrowed. It is a progressive chronic disease showing the symptoms of abdominal pain and distension, diarrhoea, anaemia, weight loss, bleeding and sepsis. Treatment is difficult and prognosis poor.

15 The 4D5.4 receptor-ligand-antibody system specifically described herein provides both diagnostic tools and therapeutic agents providing means for modulating and controlling the immune response in diseases involving activated T lymphocytes, inflammatory  
20 diseases including inflammatory gut diseases such as Crohn's disease.

Therefore, the present invention provides a binding partner (receptor) 4D5.4 as identified by reactivity with the monoclonal antibody 4D5.4. The monoclonal antibody  
25 is produced by the hybridoma 4D5.4 which has been deposited under the terms of the Budapest Treaty at ECACC (Porton Down, Salisbury, Wiltshire SP4 0JG, UK) on 19 November 1991 under accession No. 91111901. The present invention also provides the hybridoma 4D5.4, monoclonal  
30 antibodies (mAbs) produced by said hybridoma, other antibodies which react with the 4D5.4 receptor as identified above, and derivatives, functional equivalents and fragments of these antibodies.

The term antibody as used above should be construed  
35 as covering any binding member having a binding domain which reacts with the 4D5.4 receptor as identified above. Thus the invention also covers antibody fragments, derivatives, functional equivalents and homologues of the

antibody.

Example antibody fragments, capable of binding an antigen or other binding partner are (i) the Fab fragment consisting of the VL, VH, CL and CH1 domains; (ii) the Fd fragment consisting of the VH and CH1 domains; (iii) the Fv fragment consisting of the VL and VH domains of a single arm of an antibody, (iv) the dAb fragment (Ward, E.S. et al., Nature 341, 544-546 (1989) which consists of a VH domain; (v) isolated CDR regions; and (vi) F(ab')<sub>2</sub> fragments, a bivalent fragment comprising two Fab fragments linked by a disulphide bridge at the hinge region. Also, although the two domains of the Fv fragment are coded for by separate genes, it has proved possible to make a synthetic linker that enables them to be made as a single protein chain (known as single chain Fv (scFv) fragment; Bird, r.E. et al., Science 242, 423-426 (1988) Huston, J.S. et al., Proc. Natl. Acad. Sci., USA 85, 5879-5883 (1988)) by recombinant methods.

The present invention encompasses any fragment of the 4D5.4 antibody and any fragments of any ligand which binds the same binding partner as 4D5.4. Such a ligand may therefore be any antibody fragment described above.

A derivative is a substance derived from e.g. a polypeptide, ligand or antibody. The derivative may differ from a polypeptide from which it is derived by the addition, deletion, substitution or insertion of amino acids, or by the linkage or fusion of other molecules to the polypeptide. Changes such as addition, deletion, substitution or insertion may be made at the nucleotide or protein level. An example derivative is a fusion of an antibody fragment such as a Fab fragment linked to an Fc tail from another source. Other examples are linking of markers such as enzymes, fluoresceins etc. to antibody fragments.

The term "ligand" is used to denote something which is capable of binding a binding partner. It encompasses antibodies and fragments, derivatives, functional equivalents and homologues of antibodies (as described

herein). It also is used to cover the molecule for which the 4D5.4 binder partner is the natural receptor, and functional or synthetic equivalents of the molecule.

Other ligands such as antibodies which react with the 4D5.4 receptor may be identified by use of the 4D5.4 monoclonal antibodies produced by the deposited hybridoma 4D5.4. For example, the 4D5.4 monoclonal antibodies can be labelled with a detectable moiety e.g. biotin. Other useful antibodies can then be found by testing the ability of a sample suspected of containing useful antibody to displace labelled 4D5.4 monoclonal antibody from cells expressing the 4D5.4 receptor, for instance HT29 cells induced with gamma interferon (See for example, Hale et al (1987) in Leucocyte Typing Vol.III, edited by A.J. McMichael et al, Oxford University Press, p.811). Alternatively, the 4D5.4 monoclonal antibody can be used to purify the 4D5.4 receptor. The purified 4D5.4 receptor can then be used in for example, an immunoprecipitation or Western blotting technique or radioimmunoassay technique to screen for antibodies from sources other than the deposited 4D5.4 hybridoma which also react with the 4D5.4 receptor. (See for example, Dalchau et al (1987) in Leucocyte Typing Vol.III, edited by A.J. McMichael et al, Oxford University Press, p.814).

It will be understood by those skilled in the art that the hybridoma may be subject to genetic mutation or other changes, preferably while still retaining its ability to produce monoclonal antibody of the same specificity. The present invention therefore encompasses mutants and other derivatives of the hybridoma.

It will be further understood by those skilled in the art that a monoclonal antibody can be subjected to the techniques of recombinant DNA technology to produce other antibodies or chimeric molecules which retain the specificity of the original monoclonal antibody. Such techniques may involve introducing DNA encoding the immunoglobulin variable region, or the complementarity determining regions (CDRs), of the monoclonal antibody to

the constant regions, or constant regions plus framework regions, of a different immunoglobulin, for example to convert the mouse-derived monoclonal antibody into one having largely human immunoglobulin characteristics (see  
5 EP 184187A, GB 2188638A).

The Winter patent application EP-A-0 239 400 describes how it is possible to make an altered, derivative, antibody by replacing the complementarity determining regions (CDRs) of a variable region of an  
10 immunoglobulin with the CDRs from an immunoglobulin of different specificity, using recombinant DNA techniques, so called "CDR-grafting". This enables antigen-binding specificity of one antibody (in the present case it might be 4D5.4 mAb or an antibody with the same binding  
15 specificity or an antibody which is cross-reactive with 4D5.4) to be transferred to another antibody. This enables "humanisation" of antibodies. A "humanised" antibody with the CDRs of a rat or mouse antibody specific for an antigen of interest, might well be less  
20 likely to be recognised as foreign by the immune system of a human, and so be of particular use in human therapy and/or diagnostic methods.

EP-A-0 120 694 (Boss et al/Celltech) describes the cloning and expression of chimeric antibodies. In these  
25 derivatives the variable domains from one immunoglobulin are fused to constant domains from another immunoglobulin. Usually, the variable domains are derived from an immunoglobulin from one species, say a mouse or a rat, and the constant domains are derived from  
30 an immunoglobulin from a different species, perhaps a human. This technology is now very well known in the art.

A later European Patent Application, EP-A-0 125 023 (Cabilly/Genentech), also US 4816567, describes much the  
35 same subject as the Boss patent application, but describes production of other variations of immunoglobulin-type molecules using recombinant DNA technology. Another possibility is to attach just the

variable region of the monoclonal antibody to another non-immunoglobulin molecule, to produce a chimeric molecule (see WO 86/01533 Neuberger and Rabbits/Celltech). A further possibility would be to produce a chimeric immunoglobulin having different specificity in its different variable regions, one of which is that of the monoclonal antibody of the present invention (see EP 68763 A). Yet another possibility would be to produce a mutation in the DNA encoding the monoclonal antibody, so as to alter certain of its characteristics without changing its essential specificity. This can be done by site-directed mutagenesis or other techniques known in the art. All such derivatives of the monoclonal antibody hereof, are encompassed by the present invention.

The provision of the 4D5.4 receptor as identified by reactivity with monoclonal antibody 4D5.4 allows persons skilled in the art to obtain natural or synthetic ligand which bind to the receptor utilising standard techniques well known in the art. Therefore, the present invention also provides natural or synthetic ligands which bind with said 4D5.4 receptor. These ligands may be obtained according to techniques well known in the art. For example, the purified 4D5.4 receptor can be used in an immunoprecipitation or Western blotting technique to screen a sample suspected of containing a ligand for the 4D5.4 receptor. (See for instance, Armitage et al (1992) Nature 357; 80-82).

The antibodies, receptors and ligands as provided by the present invention may be used as therapeutics to control the immune response in inflammatory disorders, eg in the gastrointestinal tract.

The provision of the 4D5.4 receptor and the natural ligand enable the setting up of screening assays to look for compounds which interfere with the natural interaction. This could be done with either a cell line expressing the cell surface receptor as a result of transfection or with a cell line which naturally



expresses the receptor. This cell line would then be used with ligand which has been labelled in such a way (e.g. with a radioisotope, an enzyme or a fluorochrome) that it is possible to detect binding of the ligand to the receptor bearing cell. Samples suspected of containing a compound capable of interfering with this natural interaction would be added to the reaction mixture and inhibitory compounds would be identified as those samples causing a reduction in the binding of the labelled ligand to the receptor (see for example, Arend et al (1989) J. Immunol. 143; 1851-1858). Any such compounds may be useful as modulators of the immune response.

The provision of the 4D5.4 receptor also enables the cloning of the gene encoding the receptor according to techniques well known in the art. For example, the 4D5.4 receptor can be purified and subjected to sequence analysis. The amino acid sequence enables the provision of nucleotide coding sequences which can be used to identify the gene coding for the 4D5.4 receptor. This gene can be used in an appropriate recombinant expression system to provide cloned 4D5.4 receptor. Alternatively, messenger RNA can be isolated from either a cell line expressing the 4D5.4 receptor (e.g. RPMI 8866) or from a cell line induced to express the receptor (e.g. HT-29 cells stimulated with gamma interferon). This messenger RNA can then be used to make cDNA which is then used in an appropriate recombinant expression system in either a eukaryotic (B.Seed Nature, 1987, 329: 840-842) or prokaryotic host (R.A. Young and R.W. Davis Proc. Natl. Acad. Sci. USA, 1983, 80: 1194-1198).

Similarly, any natural ligand for the 4D5.4 receptor may be cloned.

Thus the present invention provides cloned versions of the natural 4D5.4 receptor and of its natural ligand. The present invention also provides vectors (cloning and expression vectors) incorporating the nucleotide sequences, encoding antibodies, receptors, and ligands as

discussed above, transformed cells incorporating said vectors and expression products produced by use of a recombinant system utilising any such vectors or transformed cells.

5       The present invention also provides recombinant methods for expressing antibodies, receptors and ligands as discussed above which comprises using a nucleotide sequence, vector or transformed cell as defined above.

10       The antibodies and ligands hereby provided can be used in diagnostic tests and procedures, for example, in detecting the 4D5.4 receptor, perhaps in a clinical sample derived from the body, to establish the existence or otherwise of an inflammatory disease.

15       The antibodies and ligands can also be used as imaging reagents in the diagnosis of an inflammatory condition e.g. by administering to a patient, such as one suspected of suffering from Crohn's disease or other gastrointestinal inflammatory condition, either the whole antibody or ligand or fragments thereof labelled with a  
20       radioisotope such as <sup>99</sup>Technitium or <sup>111</sup>Indium.

They can also be used therapeutically or prophylactically.

Also provided are diagnostic test kits comprising antibodies and ligands hereby provided.

25       Also provided are medicaments which comprise an antibody or ligand hereby provided for treatment of an inflammatory condition. The antibody or ligand could be delivered for instance by intravenous injection in a sterile solution of phosphate-buffered saline (see for  
30       example Isaacs et al (1992) Lancet 340; 748-752). Other modes of administration may be used, as appropriate.

The present invention also provides methods of using the antibodies and ligands hereby provided to make:

35       (a) diagnostic reagents and kits to establish the existence or otherwise of an inflammatory disease; and/or  
      (b) medicaments to treat an inflammatory condition. The present invention also provides a method of diagnosing or treating an inflammatory disease which comprises using an

antibody or ligand hereby provided.

The experimental data herein demonstrate that the addition of an antigen (eg tetanus toxoid) or mitogen (eg phytohaemagglutinin or pokeweed mitogen) to blood mononuclear cells results in the proliferation of T lymphocytes, proliferation which is significantly inhibited by the addition of 4D5.4 antibody.

The experimental data herein illustrate enteropathy similar to the damage seen in inflammatory bowel disease, generated in fetal guts in organ culture by use of a lymphocyte activator (e.g. pokeweed mitogen) to stimulate a local T cell mediated immune response, the addition of 4D5.4 antibody at the onset to organ cultures of fetal guts stimulated in this way being shown to inhibit significantly gut damage.

The experimental data herein suggest that the activation of lymphocytes such as T cells, is an early step in a likely sequence of events finally resulting in the observed enteropathy. Thus, it is thought that the addition of 4D5.4 antibody to such a fetal gut cell culture system interferes with lymphocyte activation hence preventing the enteropathy.

According to the present invention there is provided the antibody 4D5.4, obtainable from deposit ECACC 91111901, or a fragment or derivative of said antibody 4D5.4. Also provided is a ligand which binds the same binding partner as the antibody 4D5.4. Such a ligand may be an antibody, antibody fragment or antibody derivative. Alternatively, the ligand provided may be the natural ligand for the binding partner receptor, which has been identified by its reactivity with the antibody 4D5.4, or a synthetic or functional equivalent of said natural ligand.

A ligand according to the present invention may have binding specificity for an epitope to which antibody 4D5.4 is not capable of binding. For instance, the ligand may be able to bind to the 4D5.4 receptor at an epitope which is far enough from the epitope reactive

with the antibody 4D5.4 to allow binding to the receptor of both the provided ligand and the antibody 4D5.4 at the same time.

5 A ligand according to the present invention may be one which competes with antibody 4D5.4, obtainable from deposit ECACC 91111901, for binding to a binding partner. This may be because the ligand binds to the same epitope as the 4D5.4 antibody, or because the ligand binds an epitope which is so close to the epitope reactive with  
10 the 4D5.4 antibody that binding of both the ligand and the 4D5.4 antibody to the same binding partner molecule at the same time is impossible.

The present invention also provides a binding partner to which the antibody 4D5.4 is capable of  
15 binding, and fragments or derivatives of such a binding partner.

A further aspect of the present invention provides a method of obtaining a binding partner, the method comprising using an antibody or ligand according to any  
20 one of claims 1 to 5.

The hybridoma 4D5.4, deposit ECACC 91111901, and mutants, derivatives and descendants of said hybridoma are also provided by the present invention.

The antibody 4D5.4 or a fragment or derivative of  
25 said antibody 4D5.4, or a ligand which binds the same binding partner as said antibody 4D5.4, or a binding partner to which binds said antibody 4D5.4, may be used in the manufacture of an agent for use in diagnosis of an inflammatory disorder, which may be a disorder of the  
30 gut, for example Crohn's disease.

A method of diagnosing an inflammatory disorder in an individual, comprising use of the antibody 4D5.4, or a fragment or derivative of said antibody 4D5.4, or a ligand which binds the same binding partner as said  
35 antibody 4D5.4, or a binding partner which binds antibody 4D5.4, is also provided by the present invention. Such a method may comprise determining binding to a biological sample obtained from the individual.

Also provided is a diagnostic kit, for use in diagnosing an inflammatory disorder, comprising an antibody, fragment or derivative, ligand or binding partner as described herein.

5       The present invention also encompasses the use of the antibody 4D5.4, or a fragment or derivative of said antibody 4D5.4, or a ligand which binds the same binding partner as said antibody 4D5.4, or a binding partner to which binds said antibody 4D5.4, in the manufacture of a  
10       medicament for treating an inflammatory disorder, which may be a disorder of the gut, for instance Crohn's disease.

      Methods of treatment of inflammatory disorders, comprising administration of the antibody 4D5.4, or a  
15       fragment or derivative of it, or a ligand which binds the same binding partner as said antibody 4D5.4, or a binding partner to which binds said antibody 4D5.4, to an individual, are envisaged by the present invention.

      The present invention also provides use of (i) a  
20       ligand which binds a binding partner to which the antibody 4D5.4 is capable of binding and (ii) said binding partner, in identification of compounds which interfere with interaction of the receptor recognised by said antibody 4D5.4 and the natural ligand of said  
25       receptor.

      In order that the present invention is more clearly understood embodiments will now be described in more detail with reference to experimental data.

30

      4D5.4 is a mouse anti-human monoclonal antibody of the IgG2a isotype. The hybridoma able to produce it has been deposited at the European Collection of Animal Cell Cultures, Division of Biologics, Porton Down, Salisbury,  
35       Wiltshire SP4 0JG, UK on 19 November 1991 under accession number 91111901. It was prepared by immunising a Balb/c mouse with cells isolated by collagenase digestion of colonic biopsies from a patient with active Crohn's

disease and another patient with ulcerative colitis. Colonoscopies were carried out using an Olympus PCF Paediatric Colonoscope (Olympus Corporation of America, New Hyde Park, N.Y.). The specimens were taken from  
5 sites of active epithelial inflammation. The specimens were immediately placed in cold tissue culture preserving medium. Cells were isolated from the biopsies by incubating them for 2.5h at 37°C in 0.1% collagenase (Sigma Chemical Co., St. Louis, Mo.) in tissue culture  
10 medium (total volume 5ml) with vigorous pipetting every 15 min. Any small clumps of debris remaining after digestion were allowed to settle out at under gravity. Cells remaining in suspension were spun down and resuspended in a small volume of culture medium. The  
15 immunisation was carried out with the cell preparation comprising predominantly epithelial cells and some lymphocytes.

The mouse received a first dose from the Crohn's disease patient of 1 million cells intraperitoneally, was  
20 rested for 2 weeks and then received a second dose from the ulcerative colitis patient of 1 million cells intraperitoneally. Three days later, spleen cells were isolated and hybrids were made with NSO's using standard procedures. Microtiter wells showing growth were  
25 screened for reactivity against Crohn's bowel and normal bowel by immunohistochemistry on frozen sections, using peroxidase rabbit anti-mouse and diaminobenzidine reagent to visualise antibody binding. The antibody 4D5.4 was identified because it recognised epithelium from inflamed  
30 tissue, but not epithelium from normal tissue. It was then recloned by limiting dilution according to standard techniques.

The tissue reactivity of the monoclonal antibody 4D5.4 was then investigated using the standard technique  
35 of indirect peroxidase immunohistochemical staining (see e.g. Immunocytochemistry. Practical Applications in Pathology and Biology. J. Polak and S. van Noorden, editors. John Wright and Sons, Bristol, 249). Details

of tissue reactivity are given below and Table 1 summarises the information.

Tonsil: n=2

Weak staining between follicles, probably endothelium.

- 5 Some lymphocyte staining at the base of squamous epithelium.

Spleen: n=1

Staining of the cells lining of the sinusoids.

Normal blood mononuclear cells:

- 10 Negative by peroxidase.

PHA blasts:

Strongly positive on T blasts

Normal small intestine:

- 15 2 ileal biopsies-1 sample no staining, 2nd sample weak epithelial staining.

3 histologically normal sections from Crohn's disease-very patchy staining, negative epithelium in some parts, positive in other parts. Some neuronal and macrophage staining in muscle and submucosa.

- 20 Normal colon: n=4 biopsies

2 samples, no epithelial staining, 2 some weak patchy staining with positive lamina propria macrophages.

Ulcerative colitis (UC) colon: 4 biopsies and 1 resection

- 25 Epithelium very strongly positive, lamina propria macrophages and lymphoid follicles were positive.

Crohn's colon: 5 resected samples

Epithelium very strongly positive in all samples.

Neuronal cells, macrophages and lymphoid follicles were positive throughout the tissue.

- 30 Crohn's small bowel: 5 resected samples

Same as Crohn's colon

Colonic carcinomas: n=3

No epithelial staining

- 35 Titration of the antibody reveals that staining seems to be most intense on neuronal cells in diseased tissues, lymphoid staining titrates out before macrophage and epithelial staining.

Table 1

Summary of Staining in the Gut

		<u>Epithelium</u>	<u>Lamina propria</u>	<u>Follicles</u>	<u>Muscle</u>	<u>Neurones</u>
5	Normal small bowel	- or ±	- or weak macrophage	none seen	-	-
	Normal colon	- or ±	- or weak macrophage	none seen	-	-
10	Inactive Crohn's	-, ± or ++	patchy macrophages associated with epithelial staining	positive	-	± or +
15	Active Crohn's	+++++	+++++	++++	-	+++++
	Active UC	+++++	+++++	+++++	-	+++++
20	Normal fetal gut	-	-	-	-	-
	PWM-treated fetal gut	+++++	+++++	+++++	-	+++++

25

Thus the antibody is positive on Crohn's epithelium and negative on normal epithelium. Whilst one occasionally sees small patches of positive cells in a normal epithelium at sites of inflammation, the epithelium is strongly positive as are macrophages and some lymphoid cells. With high concentrations of antibody, it is felt that all macrophages showed a weak positive staining. With tonsil and spleen sections, staining is normally low or negative, but an occasional tonsil section has shown positive staining which may be related to infection in the tonsil. Normal peripheral blood lymphocytes have negative or very low levels, but in the presence of PHA the staining increased.

30

35

40

There is no staining on resting Jurkat cells (a human T cell line) but staining becomes strongly positive in the presence of a tonsil phytohaemagglutinin (PHA) supernatant.

45

The tissue reactivity of the monoclonal antibody on other tissues was studied using the standard technique of indirect peroxidase immunohistochemical staining (see e.g. Immunocytochemistry. Practical Applications in



Pathology and Biology. J. Polak and S. van Noorden, editors. John Wright and Sons, Bristol, 249). Details of staining are given below and Table 2 summarises the results.

- 5 Liver: n=2  
Some Kupfer cells weakly positive. Basal area of bile duct positive in one sample.  
Brain: n=2  
Capillary endothelium weakly positive.
- 10 Kidney: n=2  
Glomerular capillary tufts weakly positive. Some small vessels weakly positive in one sample.  
Skin: n=1  
Epidermis uniformly strongly positive. Dermis
- 15 negative.  
Breast: n=1  
Occasional macrophages positive.  
Muscle: n=1  
Negative.
- 20 Pancreas: n=1  
Small amount of connective tissue positive. Some macrophages positive.  
Thyroid: n=1  
Occasional macrophages positive.

25

TABLE 2  
Summary of staining of tissues

		<u>weak</u>	<u>moderate</u>	<u>strong</u>
30	Liver	Kupfer cells	-	-
	Brain	Capillary endo.	-	-
	Kidney	Some small vessels; glomeruli	-	-
	Skin	-	-	epidermis
35	Breast	Occasional macrophages	-	-
	Muscle	-	-	-
	Pancreas	Occasional macrophages some connective tissue	-	-

Thyroid Occasional macrophages -

Previous results with the immunoperoxidase method had shown that the 4D5.4 antibody was negative on normal blood mononuclear cells. Using a standard, more sensitive flow cytometric technique (see e.g. Callard et al, 1987 Indirect Immunofluorescence Analysis in Lymphokines and Interferons, A Practical Approach by M.J. Clemens, A.G. Morris and A.J.H. Gearing, editors. IRL press, Oxford, 352) we now observe that the 4D5.4 antibody stains all blood mononuclear cells weakly. Using the same technique it stains the lymphoid and myeloid derived cell lines HFB1, HL-60, J6, KM3, Molt4, Nalm-6, RPMI 8866, U937. It does not stain the lymphoid cell line Daudi. All the lines used are commonly available although our lines did not come directly from ATCC or ECACC. The results are summarised in Table 3.

TABLE 3  
Summary of cell staining

		<u>Staining</u>	<u>ATCC</u>	<u>ECACC</u>
	Normal blood:			
	T lymphocytes	+	-	-
25	B lymphocytes	+	-	-
	monocytes	+	-	-
	Daudi	-	CCL 213	
	HFB1	++		
	HL-60	+	CCL 240	
30	J6	+		88052401
	KM3	+++		
	Molt4	++	CRL 1582	
	Nalm-6	++		
	RPMI 8866	+++		
35	U937	+	CRL 1593	

The 4D5.4 receptor (4D5.4R) does not appear to be expressed on normal HT29 cells (a human colon carcinoma

line), but after 24 hours culture with recombinant interferon gamma (100 units/ml), up to 40-50% of the cells became positive. This effect titrates out with reducing doses of interferon.

5

TABLE 4

Dose of Interferon		% HT29+ cells expressing 4D5
	0	0
10	0.1 U/ml	1.3
	1.0 U/ml	10.9
	10 U/ml	20.8
	100 U/ml	43.4

Cells were cultured with interferon for 24 hrs.

15 HT29 cells were grown on glass coverslips and when they became dense, the coverslips were transferred into 24 well-plates containing the different concentrations of IFN. After 24 hours the coverslips were removed, air-dried, glued onto slides, acetone fixed and stained for  
20 4D5.4 expression by immunoperoxidase.

A series of experiments was performed to see what effect the 4D5.4 antibody had on in vitro T cell proliferation induced by three different agents: tetanus toxoid (TT), pokeweed mitogen (PWM) and  
25 phytohaemagglutinin (PHA). Tonsil or peripheral blood mononuclear cells (TMNC and PBMC respectively) were incubated in culture wells (200,000 cells per well in 200ul of buffer) with PHA (0.5 µg/ml) or PWM (0.5 µg/ml). The cells were incubated for three days and proliferation  
30 of the cells was assessed by measuring the incorporation of the radioactive DNA precursor tritiated thymidine. The results shown are the mean value obtained from three wells per treatment.

35

TABLE 5

		<u>Tritiated thymidine incorporation (cpm)</u>	
		<u>PHA</u>	<u>PWM</u>
40	No antibody	68,388	93,033
	Mitogen + 4D5.4 (10 ug/ml)	10,351	3,615

18

+ 4D5.4 (2.5 ug/ml)	26,143	14,379
+ 4D5.4 (0.6 ug/ml)	44,336	50,896
+ 4D5.4 (0.3 ug/ml)	69,660	76,392

5 In both cases 4D5.4 antibody inhibited  
mitogen-induced T cell proliferation. Earlier work  
investigating the effect of 4D5.4 antibody on tritiated  
thymidine uptake of blood T cells stimulated with PHA  
lead to a tentative conclusion that 4D5.4 antibody did  
10 not inhibit PHA response of the cells. However, the  
early experiments used 4D5.4 supernatant and ascites, in  
which the amount of antibody was unknown, and used a much  
higher concentration of PHA (5µg/ml) than that used in  
the experiments from which the results shown in Table 5  
15 were obtained.

In order to look at a response in vitro which more  
closely mimicked what occurs in vivo, PBMC from two  
healthy volunteers who had been immunised  
intra-muscularly 4-8 weeks previously with tetanus toxoid  
20 (Wellcome, Simple Vaccine) were set up in culture wells  
(200,000 cells per well in 200ul of buffer) with TT  
antigen (37.5 ng/ml) for 5 days. Proliferation of the  
cells was assessed by measuring the incorporation of the  
radioactive DNA precursor tritiated thymidine. The  
25 results shown are the mean value obtained from three  
wells per treatment.

TABLE 6

Tritiated thymidine incorporation (cpm)

	<u>Donor 1</u>	<u>Donor 2</u>
30 Control	401	332
TT alone (37.5 ng/ml)	24,030	12,209
TT (37.5 ng/ml)+ 4D5.4 (50 ug/ml)	1,537	6,129
+ 4D5.4 (10 ug/ml)	6,595	8,639
35 + 4D5.4 (2 ug/ml)	21,478	14,169

The results summarised in Tables 5 and 6 show that  
the 4D5.4 receptor expressed in activated T cells is

involved in T cell activation by either mitogens or antigens.

The antibody 4D5.4 does not stain human fetal small intestinal epithelium. If however, a local T cell mediated immune response is generated in fetal guts in organ culture, (the addition of lymphocyte activators such as pokeweed mitogen (PWM) to an organ culture of human fetal guts causes an enteropathy similar to the damage seen in inflammatory bowel disease) then strong 4D5.4 expression is seen on epithelium, lamina propria macrophages and T cells. However, the addition of 4D5.4 antibody at the onset to organ cultures of fetal guts stimulated with pokeweed mitogen significantly inhibits gut damage.

Small intestine from therapeutically aborted fetuses were placed in petri-dishes in serum-free CMRL-1066 medium (Flow Laboratories, Inc., McLean V.A) modified as described by Autrup et al. (Autrup H., Barrett, L.A., and Jackson F.E., et al 1978 Explant culture of human colon. Gastroenterology 74:1248). The intestine was cut into segments 2-3 mm in length which were then bisected longitudinally to expose the lumen. The explants were then trimmed into pieces 2-3 mm square for culture. Pieces of tissue were cultured in (i) 7ml modified CMRL-1066 medium; (ii) CMRL-1066 plus 4D5.4 antibody; (iii) CMRL-1066 plus PWM (pokeweed mitogen); and (iv) CMRL-1066 plus PWM plus 4D5.4 antibody in 5 cm diameter tissue culture dishes (Sterilin; Scientific Supplies Co., Ltd., Vine Hill, London). PWM was added to the appropriate test culture at a concentration of 15µg/ml, a concentration shown to be optimal in preliminary experiments. Cultures were incubated for 72 h at 37°C in a 95% oxygen 5% CO<sub>2</sub> atmosphere. At the end of the incubation period, the explants were examined on an inverted phase contrast microscope.

If villi were visible below the debris, the explant was scored as having villi. Fetal guts were 14-16 weeks old. Each experiment used a different fetal gut. This

system depends on the polyclonal activation of lamina propria T cells in fetal guts in organ culture by the lectin pokeweed mitogen. The damage caused by the cell-mediated response in the fetal guts resembles that seen in untreated coeliac disease and Crohn's disease.

The results are shown in Table 7 below.

TABLE 7				
	<u>Experiment 1</u>	<u>Villi visible</u>	<u>Enteropathy</u>	
10	Controls	6	0	0%
	4D5 alone	20	0	0%
	PWM	1	5	85%
	PWM+ 4D5 (1-10% sup)	19	1	5%
15	<u>Experiment 2</u>			
	Controls		contaminated	
	PWM	3	17	85%
	PWM + 4D5 (1&5% Asc)	22	12	36%
20	<u>Experiment 3</u>			
	Controls	7	0	0%
	PWM	1	10	90%
25	PWM + 10% 4D5 sup	7	2	18%

The results show that the presence of the antibody 4D5.4 inhibits damage in T cell mediated enteropathy of fetal gut.

#### The growth and production of the monoclonal antibody 4D5.4

As shown and discussed above, the 4D5.4 monoclonal antibody is specific to a receptor expressed in gut inflammation. The antibody is produced from a mouse x mouse hybridoma produced by the fusion of the mouse myeloma fusion partner NS0 and the spleen cells from a Balb/c mouse which had been immunised with inflamed gut epithelial cells. The hybridoma was shown to be contaminated with Mycoplasma hyorhinis, but it has been routinely cultured, cryopreserved, cloned and produced in quantity without presenting problems.

All results obtained with antibody derived from mycoplasma-contaminated hybridoma cells have been

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reproduced with antibody obtained from mycoplasma-free hybridoma cells.

Routine culture of the hybridoma

This has been achieved at 37°C in a 5% CO<sub>2</sub>, humidified incubator. In static culture, the cells were grown in 10% Foetal Bovine Serum (FBS) Dulbeccos Modified Eagles Medium (DMEM) or 10% FBS RPMI 1640 medium without antibiotics. Cells were maintained within a concentration range of  $5 \times 10^4$  to  $5 \times 10^5$ /ml.

10 Bulk roller cultures

These were established by the addition of 50ml of cells at  $3-5 \times 10^5$ /ml from static culture to an 850cm<sup>2</sup> roller bottle and then adding 200ml of complete culture medium. The roller culture was incubated at 37°C in a 5% CO<sub>2</sub> humidified incubator overnight without rotation, before transfer to a 37°C roller incubator rotating at 1 r.p.m., with the cap closed. When a cell concentration exceeding  $2 \times 10^5$ /ml had been achieved, a further 250ml of complete culture medium was added. When this 500ml culture had achieved a cell concentration of approximately  $3 \times 10^5$ /ml, the culture was either subcultured into 10 more roller bottles, in order to achieve greater volumes, or 1 litre of culture medium without FBS was added and culture continued until all cells were dead by overgrowth.

The cell concentrations which could be achieved in roller cultures were greater than those of static cultures; concentrations within the range of 2 to  $4 \times 10^6$  have been achieved.

30 Mycoplasma isolation

a. Gen Probe Mycoplasma T.C. Rapid Detection system, resulting positive.

b. Mycoplasma Experience (M.E. at Netherne Hospital, Netherne Lane, Coulsdon, Surrey CR3 1YX) Cell screen; 0.2ml of cell suspension was inoculated onto ME solid media and 2.0ml into ME arginine/glucose broth. After 5 days incubation, mycoplasma colonies were observed on the solid medium and an acid colour change

indicating glucose fermentation was produced in broth.

#### Mycoplasma Identification

This was achieved by the fact that the colony morphology of the isolate was typical of *M.hyorhinitis*,  
5 then a disc impregnated with concentrated rabbit antiserum to *M.hyorhinitis* was applied to an agar plate onto which the isolate had been subcultured, (The disc was from a batch pre-tested for specificity and activity against the target species). A zone of growth inhibition  
10 confirmed the isolate as *M.hyorhinitis*.

#### Mycoplasma Eradication

This was achieved by the cell line undergoing five passages in the presence of 1/50 dilution of specific rabbit anti-*M.hyorhinitis* antiserum and a pre-tested anti-  
15 mycoplasmal antibiotic Ciprofloxacin (10ug/ml). The cell culture medium contained no other antibiotic except Ampicillin. After treatment the cell line underwent a further five passages in antibiotic free medium (Ampicillin was permitted), and was resubmitted for  
20 mycoplasma testing. 5ml of cell suspension was tested in validated liquid and solid media. There was no mycoplasma growth within 28 days and so the cell line was considered mycoplasma free.

#### Antibody Production

25 This was achieved to a volume of 9 litres in roller culture. The yield of IgG2a 4D5.4 antibody from 8 litres of the overgrown supernatant was 180mg after Protein-A affinity chromatography. A yield of 22.5ug antibody/ml of culture supernatant. Assuming a typical 30-40% loss  
30 of antibody during downstream processing of the culture supernatant, the antibody produced in culture may be in the range of 30ug/ml.

#### Cloning of the cell line

This was achieved in 10FBS DMEM by the limiting  
35 dilution method in the presence of unstimulated peritoneal exudate cells from a Balb/c mouse, at a 30-40% cloning efficiency. Analysis of the 63 growing single colonies of the 4D5.4 cell line revealed that 95% were



producing an IgG2a antibody, the remaining 5% were not producing a detectable class of antibody and were assumed to be non-producers. The cell line had been growing for at least 20 population doublings before it was cloned.

5       The cell line, although it was contaminated with mycoplasma, may be routinely cultured giving the stable production of monoclonal IgG2a monoclonal antibody. In vitro culture yield is approximately 30ug/ml, this is very comparable to other mouse x mouse hybridomas which  
10       are not and have not been contaminated.

          Although the cloning efficiency is low in comparison to an uncontaminated line, using the established limiting dilution cloning method in the presence of mouse PEC's, single cell colonies may be achieved.

15

CLAIMS

1. The antibody 4D5.4, obtainable from deposit ECACC 91111901, or a fragment or derivative of said antibody  
5 4D5.4.
2. A ligand which binds the same binding partner as the antibody 4D5.4, antibody 4D5.4 being obtainable from deposit ECACC 91111901.  
10
3. A ligand according to claim 2 which is an antibody, antibody fragment or antibody derivative.
4. A ligand according to claim 2 or claim 3 having  
15 binding specificity for an epitope to which antibody 4D5.4 is not capable of binding.
5. A ligand according to claim 2 or claim 3 which competes with antibody 4D5.4, obtainable from deposit  
20 ECACC 91111901, for binding to a binding partner.
6. A binding partner, to which binds the antibody 4D5.4 which is obtainable from deposit ECACC 91111901.
- 25 7. A fragment or derivative of a binding partner according to claim 6.
8. A method of obtaining a binding partner according to claim 6, the method comprising using an antibody or  
30 ligand according to any one of claims 1 to 5.
9. The hybridoma 4D5.4, deposit ECACC 91111901, or a mutant, derivative or descendant of said hybridoma.
- 35 10. Use of the antibody 4D5.4, obtainable from deposit ECACC 91111901, or a fragment or derivative of said antibody 4D5.4, or a ligand which binds the same binding partner as said antibody 4D5.4, or a binding partner to

which binds said antibody 4D5.4, in the manufacture of an agent for use in diagnosis of an inflammatory disorder.

11. Use according to claim 10 wherein said inflammatory disorder is a disorder of the gut.

12. Use according to claim 11 wherein said inflammatory disorder of the gut is Crohn's disease.

13. A method of diagnosing an inflammatory disorder in an individual, comprising use of the antibody 4D5.4, obtainable from deposit ECACC 91111901, or a fragment or derivative of said antibody 4D5.4, or a ligand which binds the same binding partner as said antibody 4D5.4.

15

14. A method of diagnosing an inflammatory disorder in an individual, comprising determining the binding, to a biological sample obtained from the individual, of the antibody 4D5.4, obtainable from deposit ECACC 91111901, or a fragment or derivative of said antibody 4D5.4, or a ligand which binds the same binding partner as said antibody 4D5.4.

20

15. A method according to claim 13 or 14 wherein said inflammatory disorder is a disorder of the gut.

25

16. A diagnostic kit, for use in diagnosing an inflammatory disorder, comprising an antibody, fragment or derivative, ligand or binding partner according to any one of claims 1 to 6.

30

17. Use of the antibody 4D5.4, obtainable from deposit ECACC 91111901, or a fragment or derivative of said antibody 4D5.4, or a ligand which binds the same binding partner as said antibody 4D5.4, or a binding partner to which binds said antibody 4D5.4, in the manufacture of a medicament for treating an inflammatory disorder.

35

18. Use according to claim 17 wherein said inflammatory disorder is a disorder of the gut.

19. A method of treatment of an inflammatory disorder,  
5 comprising administration of the antibody 4D5.4,  
obtainable from deposit ECACC 91111901, or a fragment or  
derivative of said antibody 4D5.4, or a ligand which  
binds the same binding partner as said antibody 4D5.4, or  
a binding partner to which binds said antibody 4D5.4, to  
10 an individual.

20. Use of (i) a ligand which binds a binding partner to  
which the antibody 4D5.4 obtainable from deposit ECACC  
91111901 is capable of binding and (ii) said binding  
15 partner, in identification of compounds which interfere  
with interaction of the receptor recognised by said  
antibody 4D5.4 and the natural ligand of said receptor.

## INTERNATIONAL SEARCH REPORT

PCT/GB 92/02371

International Application No

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12P21/08; A61K39/395;	C07K15/00; A61K37/02	C12N5/20; G01N33/577
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12P ; C07K ; C12N ; G01N A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EP,A,O 314 863 (BAYLOR COLLEGE OF MEDICINE & BOEHRINGER INGELHEIM PHARMACEUTICALS INC.) 10 May 1989 see claims	1-20
X	EP,A,O 346 078 (THE ROCKEFELLER UNIVERSITY) 13 December 1989 see claims	1-5, 9-20
<p><sup>10</sup> Special categories of cited documents : <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
18 MARCH 1993	29.03.93	
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**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
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		JP-A- 2104534	17-04-90

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